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Skeletal Collagen Turnover by the Osteoblast

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I. Description of Research

Among the most overt negative changes experienced by man and experimental animals under conditions of weightlessness are the loss of skeletal mass and attendant hypercalciuria. These clearly result from some disruption in the balance between bone formation and bone resorption (i.e. remodelling) which appears to be due to a decrease in the functions of the osteoblast. In the studies funded by this project, the clonal osteoblastic cell line, UMR 106-01, has been used to investigate the regulation of collagenase and tissue inhibitors of metalloproteases (TIMPs). This project has shed light on the comprehensive role of the osteoblast in the remodelling process, and, in so doing, provided some insight into how the process might be disrupted under conditions of microgravity.

Cloning and Regulation of Rat TIMP-2. Together with Dr. John Jeffrey, the rat collagenase inhibitory activities secreted by the UMR 106-01 cells were purified and shown to be two tissue inhibitors of metalloproteinase (TIMP) molecules of 20 and 30 kDa. Amino-acid sequence of the inhibitor molecules from medium conditioned by the rat UMR cells showed that one of these is clearly homologous to TIMP-1 in other species. The second, and predominant, inhibitor molecule produced by UMR 106-01 cells has been identified as rat TIMP-2, by virtue of its identity to human TIMP-2 in the amino terminal region. We screened a rat genomic library with several different degenerate oligonucleotide probes synthesized to known sequences of rat TIMP-2 and obtained a positive clone. However, in addition, we obtained a human TIMP-2 cDNA which successfully hybridized to RNA from UMR cells. This indicated that there was enough sequence homology between the two sequences to use it as a probe to clone rat TIMP-2. We used this to screen our UMR cDNA library and obtained numerous positive clones. These were then sequenced and confirmed that we have obtained a rat TIMP-2 clone which has 98% amino acid sequence identity with human TIMP-2.

Parathyroid hormone (PTH), at 10^{-8} M, stimulated an approximately 2 fold increase in both the 4.2 kb and 1.0 kb transcripts over basal levels in UMR cells after 24 h of exposure. The PTH stimulation of TIMP-2 transcripts was not affected by the inhibitor of protein synthesis, cycloheximide (10^{-5} M), suggesting a primary effect of the hormone. This is in contradistinction to regulation of interstitial collagenase by PTH in these same cells. Nuclear run-on assays demonstrated that PTH caused an increase in TIMP-2 transcription which paralleled the increase in message levels. Parathyroid hormone, in its stimulation of TIMP-2 mRNA, appears to act through a signal transduction pathway involving protein kinase A (PKA) since the increase in TIMP-2 mRNA was reproduced by treatment with the cAMP analogue, 8-bromo-cAMP (8BrcAMP, 5 x 10^{-3} M). The protein kinase C (PKC) and calcium pathways do not appear to be involved due to the lack of effect of phorbol 12-myristate 13-acetate (PMA, 2.6 x 10^{-6} M) and the calcium ionophore, ionomycin (Iono, 10^{-7} M), on TIMP-2 transcript abundance. In this respect, regulation of TIMP-2 and collagenase in osteoblastic cells by PTH are similar. However, we conclude that since stimulation of TIMP-2 transcription is a primary event, the PKA pathway must be responsible for a direct increase in transcription of this gene.

Regulation of Collagenase Gene We demonstrated that stimulation of collagenase by PTH involves a substantial increase in collagenase mRNA *via* transcription. We have also concluded that this effect occurs primarily through the cAMP-dependent pathway in UMR cells and requires the expression of other genes. Our next goals were to identify the regulatory regions of the rat collagenase gene. To do this, we obtained a genomic clone containing 6.5 kbp of sequence 5′ of the transcriptional start site. This region has been subcloned upstream of the reporter gene, chloramphenicol acetyl transferase (CAT) and processive deletions made to determine the minimum amount of sequence necessary for PTH induction. So far, PTH regulation is maintained even with deletion to only 148 bases of sequence upstream of the transcriptional start site. The largest of these constructs has been transfected stably into UMR cells and will be characterized. These cells could be used in flight experiments to assess changes in transcriptional rate of the collagenase gene under microgravity conditions.

To assess the signal transduction pathway involved in PTH regulation of collagenase gene expression, we investigated changes in immediate early gene expression after PTH treatment. We demonstrated that c-*fos* and c-*jun* mRNA levels are increased 20-fold and 2-5-fold 30 min after addition of PTH. These effects precede the changes in transcription of collagenase suggesting that these transcription factors may be the proteins which induce transcription of the collagenase gene.

We have also examined regulation of collagenase in UMR cells by a series of prostanoids as well as retinoic acid. Of the former, PGE_2 was the most effective, reflecting the likelihood that the cAMP pathway is a major regulator of expression of this gene in UMR cells. Retinoic acid also stimulated collagenase expression by UMR cells. In both cases, the agents increased transcription of the MMP-1 gene and protein synthesis was required for their effects. Thus, all the hormones investigated, so far, appear to regulate collagenase by a secondary response pathway requiring expression of some other gene.

Bone Cell Research (BCR) Experiment on Spacelab-Japanese (SL-J) Mission We were recruited to Spacelab-J in November, 1990, and asked to test whether my rat osteoblastic cells, UMR 106-01, would adhere and proliferate normally in the NASDA cell culture hardware for the SL-J mission. After many preliminary growth experiments, we found that with some manipulations cells are viable, will grow and generally appear normal throughout the period which was planned for space flight in this hardware.

Many other pre-flight experiments were also conducted in a very short time. For instance, to alleviate the problem of lack of CO_2 in the Spacelab incubator, we tested, and found suitable, a CO_2-independent medium from Gibco. The cells were also tested to determine whether they would resist the effects of vibration due to lift-off and showed no problems. We also demonstrated that the hormone we were to use, PTH-related peptide, is quite stable to freezing, thawing and incubation at 4°C for an extended period of time. We also examined the stability of collagenase in medium at various temperatures and resolved that the enzyme remains unchanged at 15°C for 4 days, while at 25°C, the enzyme is stable for 24 and 48 h, but is degraded by incubation at this temperature for 72 h. These criteria established our limitations for storage of our collected media samples on the Shuttle. Many ELISA assays were conducted to test each of these parameters. In May, 1992, Cheryl Johanns, my technician, was able to participate in a Science Simulation at KSC which involved handover and scrub turnaround procedures. It was only at this time that we learned the protocol for cleaning and seeding the chambers from the Japanese investigators. Before this, we had only been provided with very limited written notes on the use of the chambers which had led to us developing procedures which may have not been optimal.

At about the same time, the flight chambers were sent to us to bio-condition and were used in a full simulation in the lab. We also sent chambers with growing cells for a crew simulation in which media exchange and photography was done. In the lab we did numerous photography sessions to determine the methods that should be followed for good photomicroscopy of the cells.

Launch was set for September 11, 1992. The launch was only a day late (with plenty of notice) and most of the flight went smoothly. The Shuttle landed at KSC which meant we received our samples within three hours of landing and they were in very good condition. The limitations on the amount of flight-certified hardware led to us not being able to conduct the best ground controls. We conducted further ground control experiments in the same flight chambers as were used on SL-J since the ones performed during SL-J were not in optimal hardware. There was a misinterpretation by one of the astronauts of the instructions on the first medium exchange which led to many mitotic cells being sloughed off the chamber surface. This resulted in far less cells being present than we anticipated. This has made it difficult to measure collagenase produced in the medium since the cell to medium ratio is so low. However, we were able to concentrate the media before assaying the enzyme by our ELISA.

The photos taken by the astronauts are extremely good and we think there are slight differences in the morphology of the flight cells compared to our ground controls. Overall, we would have to conclude that this hardware has severe limitations. The large size limits the number of chambers which can be tested and the syringes are not ideal for media exchanges. Also, the surface must be treated for use by various cells. Our general experience of a flight experiment is that more time is needed between being recruited to a mission and flight so that best use of the hardware can be determined sooner, also complete training of the astronauts needs to be accomplished.

We were unable to measure rat TIMPs by an ELISA since the affinities of the antisera we obtained were insufficient and also cross-reacted with bovine TIMPs (abundant in fetal bovine serum, FBS). The alternative method would be to measure the TIMPs by a functional assay but this was also not possible since the BCR media samples contained 10% FBS. This problem would be exacerbated by concentration of the media 50-fold.

In conclusion, there appear to be some possible morphological changes in the cells under microgravity but we are unable to determine whether these are due to microgravity or the conditions of lift-off and/or vibration. This would require an on-board 1-g centrifuge control to assess this.

Expression of Collagenase in Normal Differentiating Osteoblasts

In collaboration with Drs. Jane Lian and Gary Stein, we have found that collagenase is expressed in normal differentiating rat osteoblasts. Expression of collagenase is greatest in the most differentiated cells at a time of greatest formation of mineralized nodules. We have now examined in more detail this appearance of collagenase in normal, mineralizing, differentiating osteoblastic cultures. Collagenase is just detectable in the early part of the culture, 7 and 14 days, but then starts to increase at day 21 reaching high levels at 28 days, then declining at later ages (35 days). This profile is similar to the changes in osteopontin and osteocalcin in this type of differentiating osteoblast system. Thus, collagenase is expressed in these cultures as a late-differentiation gene which likely is involved in matrix maturation.

We have examined the effect of mineralization of the matrix on the expression of collagenase in these cultures. Basal levels of collagenase were expressed in both sets of cultures but only the group with enhanced mineralization demonstrated the late differentiation surge in collagenase. We have also measured cell growth under these two sets of conditions and the cells cultured in MEM continue to proliferate throughout the culture period, developing layers of cells; the cells in BGJ_b medium cease proliferation. Thus, several factors could be responsible for the stimulation of expression of collagenase: The cessation of proliferation could be responsible for the increase in collagenase expression. However, this seems unlikely because, proliferation ceases at 7 days but the greatest increases in collagenase occur 14 days later. Secondly, the increase in mineralization could initiate the upturn in collagenase mRNA levels. However, like proliferation, this also is initiated as soon as the cell are transferred to the mineralization medium. What is striking is that the increase in collagenase generally occurs as alkaline phosphatase is declining. Presumably, one set of transcription factors is responsible for elevation of alkaline phosphatase and levels of these, in turn, may decrease in line with decrease in alkaline phosphatase expression. This could be a signal for production of another set of transcription factors or the decline itself may be a signal for increased collagenase transcription.

We have also shown that collagenase in these cultures can be stimulated by PTH treatment. However, PTH cannot stimulate expression until basal expression is initiated.

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